# Reduced Fibulin-2 Contributes to Loss of Basement Membrane Integrity and Skin Blistering in Mice Lacking Integrin α3β1 in the Epidermis

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Deficient epidermal adhesion is a hallmark of blistering skin disorders and chronic wounds, implicating integrins as potential therapeutic targets. Integrin α3β1, a major receptor in the epidermis for adhesion to laminin-332 (LN-332), has critical roles in basement membrane (BM) organization during skin development. In the current study we identify a role for α3β1 in promoting stability of nascent epidermal BMs through induction of fibulin-2, a matrix-associated protein that binds LN-332. We demonstrate that mice lacking α3β1 in the epidermis display ruptured BM beneath neo-epidermis of wounds, characterized by extensive blistering. This junctional blistering phenocopies defects reported in newborn α3-null mice, as well as in human patients with α3 gene mutations, indicating that the developmental role of α3β1 in BM organization is recapitulated during wound healing. Mice lacking epidermal α3β1 also have reduced fibulin-2 expression, and fibulin-2-null mice display perinatal skin blisters similar to those in α3β1-deficient mice. Interestingly, α3-null wound epidermis or keratinocytes also show impaired processing of the LN-332 γ2 chain, although this defect was independent of reduced fibulin-2 and did not appear to cause blistering. Our findings indicate a role for integrin α3β1 in BM stability through fibulin-2 induction, both in neonatal skin and in adult wounds.

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## INTRODUCTION

Integrins are αβ heterodimeric, transmembrane proteins that function as the major receptors for cell adhesion to the extracellular matrix (ECM; Hynes, 1992). The laminin-binding integrins, α3β1 and α6β4, are abundant in basal keratinocytes of the epidermis, where they mediate adhesion to the basement membrane (BM), which separates the epidermis from the dermis. In unwounded epidermis, basal keratinocytes adhere to the ECM primarily through α6β4, a component of hemidesmosomes that link the keratin intermediate filaments inside the cell to anchoring filaments in the underlying BM zone ( Stepp *et al.*, 1990; Litjens *et al.*, 2006). In contrast, α3β1 localizes to actin-associated adhesion sites, which manifest as focal adhesions in cultured keratinocytes and other cells (Carter *et al.*, 1990; Grenz *et al.*, 1993; DiPersio *et al.*, 1995), and *in vivo* it promotes epidermal adhesion primarily by maintaining BM integrity as opposed to keratinocyte anchorage *per se* (DiPersio *et al.*, 1997, 2000b).

Laminin-332 (LN-332) is the main adhesive ligand in the epidermis for integrins α3β1 and α6β4 (Delwel *et al.*, 1994; Nguyen *et al.*, 2000). LN-332 is composed of three distinct chains designated α3, β3, and γ2 (Aumailley *et al.*, 2003, 2005). In resting adult skin, LN-332 lacks the C terminus of the α3 chain and the N terminus of the γ2 chain due to proteolytic processing during BM maturation (Marinkovich *et al.*, 1992; Amano *et al.*, 2000; Tsubota *et al.*, 2000; Sasaki *et al.*, 2001). Although the functional importance of this processing is not fully understood, it may regulate BM architecture by modulating interactions of LN-332 with other ECM components (Aumailley *et al.*, 2003). For instance, the L4 module within the N terminus of the unprocessed γ2 chain seems to be required for LN-332 incorporation into BM (Gagnoux-Palacios *et al.*, 2001). Notably, the L4 module contains binding sites for other ECM components, most of which are lost upon γ2 chain processing (Sasaki *et al.*, 2001), suggesting ECM linkages that may be necessary for stable incorporation of LN-332 into BM, but dispensable in mature BM. Interestingly, the laminin-γ2 chain contains two binding sites within the L4 module for the microfibrillar and BM protein, fibulin-2 (Utani *et al.*, 1997), which are lost upon proteolytic processing (Sasaki *et al.*, 2001), suggesting that fibulin-2 binding to LN-332 may regulate BM assembly.

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*Abbreviations: α3eKO, integrin α3 epidermal knockout; BM, basement membrane; ECM, extracellular matrix; LN-332, laminin-332; MK, mouse keratinocyte; shRNA, short hairpin RNA; WT, wild type*

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Mutations in the genes encoding individual chains of LN-332, or either subunit of integrin α6β4, have been linked to different forms of the inherited human blistering disorder junctional epidermolysis bullosa (Pulkkinen *et al.*, 1994a, b; Kivirikko *et al.*, 1995; Pulkkinen *et al.*, 1997; Takizawa *et al.*, 1997). In these cases, blistering is caused by detachment of basal keratinocytes from the BM, as evidenced by localization of laminin exclusively to the dermal sides of blisters. This blistering phenotype is recapitulated in mice that are homozygous for null mutations in α6, β4, or the laminin-γ2 chain (Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; van der Neut *et al.*, 1996; Meng *et al.*, 2003). Deletion of the integrin α3 subunit in mice also causes neonatal skin blisters, which is accompanied by BM disorganization (DiPersio *et al.*, 1997). However, in contrast with α6β4-deficient or LN-332-deficient mice, α3β1-deficient mice show distribution of LN-332 and other BM proteins to both dermal and epidermal sides of blisters, indicating rupture within the plane of the BM rather than keratinocyte detachment from LN-332 (DiPersio *et al.*, 1997). Indeed, distribution of LN-332 to the top sides of blisters in α3-null mice is due to retention of α6β4-mediated attachment, as LN-332 is detected only at the dermal side of blisters that form in mice lacking both α3β1 and α6β4 (DiPersio *et al.*, 2000b). Importantly, integrin α3 mutations have recently been described in humans (Has *et al.*, 2012; Nicolaou *et al.*, 2012), and patients with integrin α3-null mutations display skin blisters with striking similarities to those in α3-null mice in that LN-332 localizes to both sides of blisters (Has *et al.*, 2012), indicative of BM rupture and distinct in this way from other forms of junctional epidermolysis bullosa.

Although early studies in α3-null mice revealed a crucial role for α3β1 in maintaining BM integrity during skin development, the perinatal lethality of these mice precluded wound-healing studies. In the current study we used viable, epidermis-specific integrin α3 epidermal knockout (α3eKO) mice to evaluate post-developmental roles for α3β1 in BM organization and epidermal adhesion. Our findings indicate that α3β1 promotes stability of the neonatal epidermal BM and epidermal–dermal adhesion at least partly through induction of fibulin-2. They further show that this developmental role for α3β1 in maintaining BM integrity and epidermal adhesion is recapitulated in the regenerating epidermis of adult wounds. Interestingly, both α3β1-deficient wound epidermis and α3-null keratinocytes also showed impaired processing of the γ2 chain of LN-332. However, this phenotype was not detected in neonatal skin and it did not appear to be directly caused by reduced fibulin-2 or necessary for blistering.

## RESULTS

### Neonatal mice that lack α3β1 integrin in the epidermis display blistering in paw skin but recover by adulthood

Global deletion of the integrin α3 subunit in mice results in disorganization of the epidermal BM during skin development, leading to the formation of neonatal skin blisters caused by BM rupture (DiPersio *et al.*, 1997). These blisters are restricted to the paws, likely due to increased friction in that area (DiPersio *et al.*, 1997). It was recently shown that skin blisters

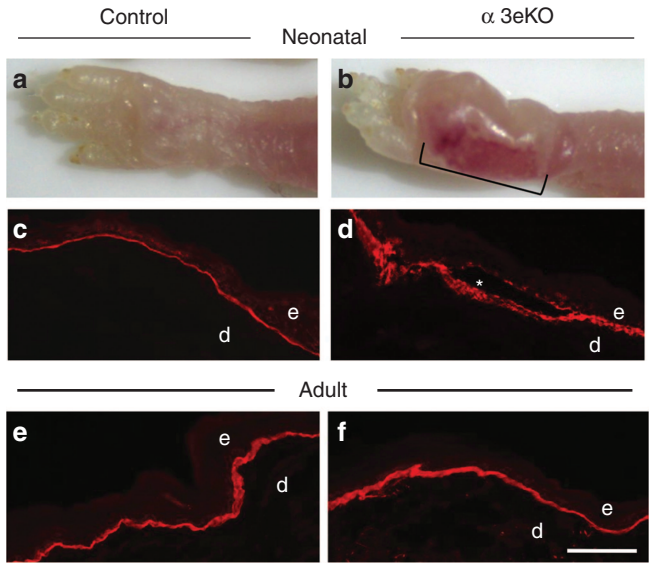


**Figure 1. Integrin α3 epidermal knockout (α3eKO) neonatal mice develop blisters in paw skin but recover by adulthood.** (**a**, **b**) Images of P2 paws from a control (**a**) or α3eKO (**b**) mouse. Bracket in **b** indicates a blood-filled blister. Cryosections of paw skin from control (**c**, **e**) or α3eKO (**d**, **f**) mice were stained by immunofluorescence with anti-laminin-332 (LN-332) to visualize the basement membrane. Sections are from P0 pups (**c**, **d**) or adult mice (>6 weeks of age; **e**, **f**). Images are from representative mice of each genotype. Blisters were detected in P0 paws of 10/11 α3eKO mice, but not in P0 control mice ($n=10$) or in adult mice of either genotype (control, $n=4$; α3eKO, $n=4$). Bar = 100 μm (applicable to **c**–**f**). d, dermis; e, epidermis; *, blister.

form in human patients with mutations in the *ITGA3* gene (which encodes the integrin α3 subunit; Has *et al.*, 2012). The onset of skin fragility was earlier in α3eKO mice than in human patients, possibly because of perinatal compensatory mechanisms that exist in humans, but not in mice. Nevertheless, human blisters bear remarkable similarities to those that form in α3-null mice (Has *et al.*, 2012). Similar to α3-null mice, these patients developed skin blisters in which LN-332 localizes to both the epidermal and dermal sides, indicative of BM rupture. These similarities suggest that α3β1-deficient mice provide a useful genetic model for the blistering component of the human disorder. However, α3-null mice die shortly after birth, making it difficult to investigate post-developmental roles for α3β1. Therefore, we used α3eKO mice in which Cre recombinase is driven by the keratin 14 promoter to effect deletion of the floxed α3 subunit gene preferentially in basal epidermal keratinocytes (Mitchell *et al.*, 2009). Epidermal deletion of α3 caused skin blisters in paws of α3eKO neonates (Figure 1a–d), attributing this phenotype to loss of α3β1 within the epidermal compartment. Importantly, LN-332 localized to both sides of these blisters (Figure 1d, asterisk), suggesting that blisters are generated via a similar mechanism as in global α3KO mice (DiPersio *et al.*, 1997) or human patients with *ITGA3* gene mutations (Has *et al.*, 2012). Entactin/nidogen, a BM component that is contributed by mesenchymal cells (Thomas and Dziadek, 1993), also localized to both sides of α3eKO blisters, further indicating BM rupture (Supplementary Figure S1 online). Neonatal paw

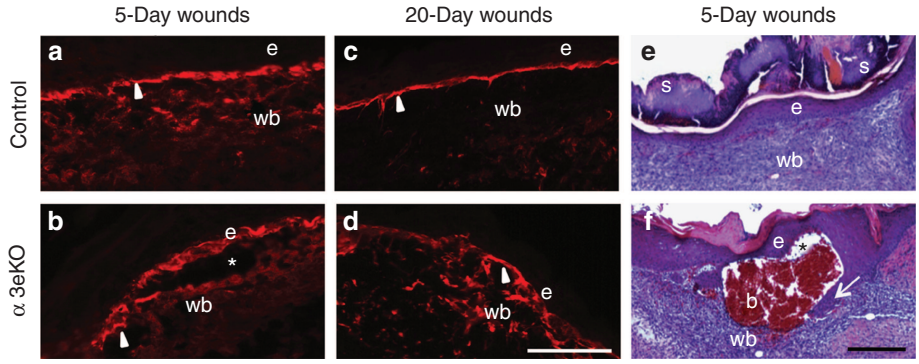


**Figure 2. Wounds of adult integrin α3 epidermal knockout (α3eKO) mice display junctional blisters and persistent basement membrane disorganization.** Cryosections of excisional wounds from control (**a**, **c**) or α3eKO (**b**, **d**) mice, isolated 5 days (**a**, **b**) or 20 days (**c**, **d**) post wounding, were stained by immunofluorescence with anti-laminin-332 (LN-332). Paraffin sections from 5-day excisional wounds of a control (**e**) or α3eKO (**f**) mouse were stained with hematoxylin–eosin. Blistering was detected in 5-day wounds of 10/12 α3eKO mice, but not in 5-day wounds of control mice ($n = 8$). Blistering was not detected in 20-day wounds of either genotype (control, $n = 4$; α3eKO, $n = 4$). White bar = 100 μm (in **a**–**d**); black bar = 200 μm (in **e**, **f**). Arrow, migrating epidermis; arrowhead, basement membrane zone (BMZ); b, blood; e, epidermis; s, eschar; wb, wound bed; *, blister.

blisters could often be seen macroscopically, sometimes with subcutaneous hemorrhaging (Figure 1b). Remarkably, we did not detect blisters in paws of adult α3eKO mice, which showed only minimal LN-332 disorganization at the BM (Figure 1e and f). These observations are consistent with a previous report that only minor microblisters were detected in the skin of adult α3eKO mice (Margadant *et al.*, 2009) and they indicate that adult mice recover from the developmental blistering defect caused by the absence of α3β1 (DiPersio *et al.*, 1997).

### The blistering phenotype observed in neonatal α3eKO mice is recapitulated during adult wound healing

It was previously demonstrated that mice lacking α3β1 in the epidermis displayed slightly faster wound re-epithelialization (Margadant *et al.*, 2009). Consistently, we observed that α3β1 was not required for re-epithelialization of adult wounds in our model (our unpublished data). As BM assembly also occurs during wound re-epithelialization, we hypothesized that α3β1 may regulate assembly of a stable BM during wound healing, thus recapitulating its developmental role. To test this hypothesis, we examined skin sections prepared 5 days or 20 days after wounding from either control (i.e., $\alpha3^{flx/flx}$; no Cre) or α3eKO mice. Five-day wounds of control mice displayed moderate LN-332 disorganization that was characterized by ectopic expression beneath the BM zone (Figure 2a), presumably reflecting deposition of newly synthesized LN-332 that is not yet assembled into BM. Nevertheless, the newly formed epidermis was in continuous contact with the underlying wound bed (Figure 2e). By 20 days post wounding, most LN-332 disorganization had resolved and the BM appeared restored to its organized state (Figure 2c). In marked contrast with control mice, fully re-epithelialized 5-day wounds of α3eKO mice displayed frequent blistering characterized by BM splitting (Figure 2b), indicating that α3β1 is required for the assembly of a stable BM. Interestingly, although LN-332 disorganization persisted in 20-day α3eKO wounds, blisters were not detected at this later stage, indicating that early wound blisters ultimately resolved (Figure 2d), similar to the recovery seen in post-developmental skin (Figure 1). Consistently, we observed that some blistered wounds displayed neo-epidermal tongues that formed beneath the blister (Figure 2f), indicating a second round of re-epithelialization that presumably was incited by the trauma of blistering. A similar mode of blister resolution has been reported in suction blisters (Leivo *et al.*, 2000).

### Laminin-γ2 chain processing is delayed in wounds of α3eKO mice

LN-332 is secreted by keratinocytes as a heterotrimer of three chains, α3 (190–200 kDa), β3 (140 kDa), and γ2 (155 kDa), and differential processing of the α3 and γ2 chains has been linked to changes in stable adhesion and migration (Gianelli *et al.*, 1997; Goldfinger *et al.*, 1998). As processing of the γ2 chain has been linked to stable incorporation of LN-332 into BMs (Aumailley *et al.*, 2003), we reasoned that epidermal α3β1 might modulate laminin-γ2 processing. To test this hypothesis, we used an antibody directed against the globular L4 module (anti-γ2L4m) of the laminin-γ2 chain (Sasaki *et al.*, 2001). As this domain is cleaved off during γ2 chain processing, positive staining indicates the presence of the unprocessed, precursor form of LN-332. To assess differential laminin-γ2 processing, we performed immunofluorescence of wounds from adult control and α3eKO mice at various timepoints post wounding. Unwounded skin of both control and α3eKO mice showed weak anti-γ2L4m staining (Figure 3a and b) compared with total LN-332 (see Figure 1), indicative of LN-332 processing. Five-day wounds of both control and α3eKO mice displayed accumulation of unprocessed laminin-γ2 at the BM zone (Figure 3c and d). Interestingly, accumulation was resolved in 10-day wounds of control animals (Figure 3e) but persisted in 10-day wounds of α3eKO mice (Figure 3f), suggesting a delay in LN-332 processing in the absence of α3β1.

α3β1 dependence of laminin-γ2 processing was further assessed by immunoblotting of matrix deposited by a panel of mouse keratinocyte (MK) cell lines, including wild-type (WT)

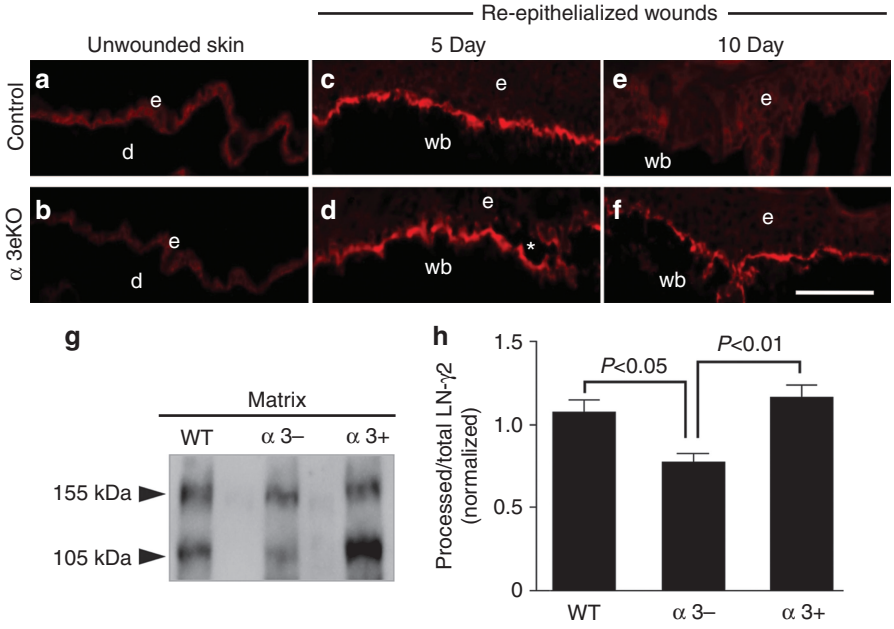


**Figure 3. Integrin α3 epidermal knockout (α3eKO) wounds display persistent accumulation of unprocessed laminin-γ2 in the basement membrane zone.** Representative cryosections of adult unwounded skin (**a**, **b**), 5-day excisional wounds (**c**, **d**), or 10-day excisional wounds (**e**, **f**) were prepared from control (**a**, **c**, **e**) or α3eKO (**b**, **d**, **f**) mice and stained by immunofluorescence with anti-γ2L4m (specific for the L4 module of the laminin-γ2 chain). Bar = 100 μm. e, epidermis; d, dermis; wb, wound bed; *, blister. (**g**) Extracellular matrix (ECM) fractions were collected from α3β1-expressing keratinocytes (wild type (WT)), α3-null keratinocytes (α3 −), or α3 − cells with restored α3 subunit expression (α3 +), cultured in high calcium (see Materials and Methods) and assessed by immunoblot with anti-laminin-γ2. The unprocessed (155 kDa) and processed (105 kDa) forms of laminin-γ2 are indicated. (**h**) Quantification of processed laminin-γ2 as a proportion of total laminin-γ2, normalized to the daily mean to account for variability by day. Data are mean ± SEM; $n = 4$; one-way analysis of variance, $P < 0.01$; Tukey's multiple comparison.

cells derived from a WT mouse, α3 − cells derived from an α3-null mouse, and α3 + cells in which α3β1 expression was restored in α3 − cells through stable transfection with human α3 (DiPersio *et al.*, 2000a; Iyer *et al.*, 2005). Previously, we reported that these cells deposit only the unprocessed 155 kDa form of laminin-γ2 when cultured in low-calcium medium (DiPersio *et al.*, 2000a), as also reported in primary keratinocytes (deHart *et al.*, 2003). Here we cultured MK cells in high-calcium medium, which others have shown induces LN-332 processing in keratinocytes (Amano *et al.*, 2000). Consistently, we detected the processed 105 kDa fragment of laminin-γ2 in matrix fractions from WT cells cultured for 3–4 days under these conditions (Figure 3g). We consistently observed reduced deposition of total laminin-γ2 in α3-null cell cultures (see Figure 5b). Importantly, we detected a significant reduction in the proportion of laminin-γ2 that is processed in the deposited matrix of α3-null cells compared with α3-containing WT or α3 + cells (Figure 3g and h), consistent with our *in vivo* data (Figure 3a–f). Although we occasionally detected enhanced processing in α3 + cells compared with WT, this difference was not statistically significant over several experiments (Figure 3g and h).

However, it seems unlikely that loss of α3β1-dependent laminin-γ2 processing directly caused blistering *in vivo*, as unprocessed laminin-γ2 accumulated by 5 days in wounds of both α3eKO and control mice (Figure 3c and d), by which time blistering was already evident in α3eKO wounds (Figure 2). In addition, neonatal blisters in α3eKO mice did not show accumulation of unprocessed laminin-γ2 (Supplementary Figure S2 online), suggesting that blistering is not dependent on reduced γ2 processing.

### Fibulin-2-null neonatal mice display skin blisters

An alternative explanation for BM splitting in α3eKO skin is that interactions of LN-332 with other essential BM components are compromised. The unprocessed laminin-γ2 short arm contains several domains for ECM interaction, some of which may be required for BM assembly and maturation. Fibulin-2 binds to the laminin-γ2 short arm in several regions, including the L4 module that is lost on proteolytic processing (Sasaki *et al.*, 2001). Interestingly, a concurrent microarray study to identify α3β1-dependent genes revealed that fibulin-2 expression is reduced in α3- cells, compared with WT or α3 + cells (Missan D and DiPersio CM, in preparation), which we have confirmed by immunoblotting (e.g., see Figure 5a). We therefore hypothesized that reduced levels of fibulin-2 in α3eKO mice may contribute to BM destabilization, resulting in blisters. To determine whether the absence of fibulin-2 is sufficient to cause blistering, we examined skin sections from neonatal mice with a global KO of the fibulin-2 gene (Sicot *et al.*, 2008). Indeed, we observed junctional skin blisters in fibulin-2-null pups (Figure 4b, asterisks), indicating that loss of fibulin-2 compromises epidermal–dermal adhesion.

### Fibulin-2 is reduced in the skin of α3eKO neonates and early wounds, but it is upregulated post blistering

Given that absence of fibulin-2 caused skin blistering (Figure 4a and b), we next asked whether fibulin-2 levels are

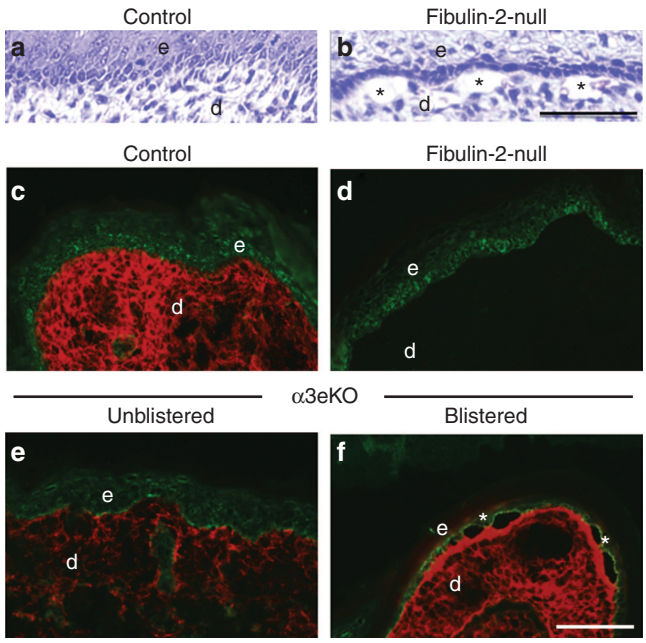


**Figure 4. Absence of fibulin-2 causes skin blistering in neonatal mice.** Hematoxylin and eosin staining of paraffin sections from paws of control (**a**) or fibulin-2-null (**b**) mice revealed blisters at the epidermal–dermal junction in the latter ($n = 6$; representative images from P0 paws are shown). Cryosections of P2 neonatal paw skin from control (**c**), fibulin-2-null (**d**), or integrin α3 epidermal knockout (α3eKO) (**e**, **f**) mice were stained by double-label immunofluorescence with anti-fibulin-2 (red) and anti-keratin 14 to mark basal keratinocytes (green). Representative images of an α3eKO paw show an unblistered region (**e**) and a blistered region (**f**). Fibulin-2 is reduced in nonblistered skin of neonatal α3eKO mice, but is upregulated in blistered regions. Reactivity to anti-fibulin-2 was not detected in fibulin-2-null mice (**d**; non-blistered region shown), demonstrating specificity. Bars = 100 μm. d, dermis; e, epidermis; *, blister.

altered in the skin of α3eKO mice. Indeed, immunofluorescence revealed that staining for fibulin-2 was reduced in neonatal α3eKO skin compared with control skin (compare Figure 4c and e). As a control, fibulin-2 staining was absent from skin of fibulin-2-null mice (Figure 4d). Fibulin-2 staining in resting adult skin was also reduced in α3eKO mice below basal levels in control mice (Supplementary Fig. S3a and b online). Interestingly, fibulin-2 was upregulated within a day of wounding in control mice (Supplementary Figure S3e online), as described previously (Fassler *et al.*, 1996), but to a much lesser extent in α3eKO mice (Supplementary Figure S3f online). Taken together, results in Figure 4 and Supplementary Figure S3 online indicate that expression of α3β1 maintains fibulin-2 levels during skin development and wound repair, consistent with a role in BM integrity and epidermal–dermal adhesion.

Lack of extensive blistering in resting skin of adult α3eKO mice indicates that these mice somehow overcome the skin blistering incurred as neonates (Figure 1; Margadant *et al.*, 2009). As described above, blisters that formed in 5-day, re-epithelialized wounds of α3eKO mice appeared to incite a second round of re-epithelialization (Figure 2f), and blisters were not detected in re-healed wounds of these mice (Figure 2d), suggesting a compensatory adhesion mechanism during the second round of re-epithelialization. Interestingly, we observed that fibulin-2 was eventually upregulated in α3eKO wounds to a similar level as in control wounds by 5 days post wounding (Supplementary Figure S3g and h online), at which time wound blistering was already detected (Figure 2). Similarly, fibulin-2 levels were enhanced in blistered regions of α3eKO neonatal skin, particularly in the area proximal to the epidermis (Figure 4f). These observations suggest that junctional skin blistering in α3eKO mice triggers compensatory upregulation of fibulin-2 via an unknown mechanism that is independent of α3β1, which might contribute to successful blister resolution in adult skin and re-healed wounds of these mice.

### α3β1-dependent modulation in laminin-γ2 processing is independent of fibulin-2

Given that absence of α3β1 from wound epidermis leads to delays in both laminin-γ2 processing (Figure 3) and fibulin-2 expression (Supplementary Figure S3 online), we next asked whether laminin-γ2 processing is altered upon suppression of fibulin-2. WT cells stably infected with lentivirus expressing either of two short hairpin RNAs (shRNAs) that target fibulin-2 mRNA showed decreased fibulin-2 protein of 40 or 60% compared with non-targeting control shRNA (Figure 5a), with the more effective shRNA reducing fibulin-2 nearly to levels seen in control-infected α3-null cells (Figure 5a). However, shRNA-mediated suppression of fibulin-2 in WT cells did not detectably alter laminin-γ2 expression in whole-cell lysates or processing in deposited matrix, indicating that α3β1-dependent processing of laminin-γ2 is not dependent on fibulin-2 levels (Figure 5b).

### Blisters of fibulin-2-null mice and α3eKO mice heal in a similar manner

Similar to adult α3eKO mice, adult fibulin-2-null mice were not reported to display obvious skin blisters, indicating that blisters heal postnatally, perhaps due to compensation by other fibulin family members for the global absence of fibulin-2 (Sicot *et al.*, 2008). We therefore examined skin of either fibulin-2-null or α3eKO pups that were several days old, in order to assess post-natal blister resolution. Interestingly, in the skin of both fibulin-2-null pups (P2) and α3eKO pups (P10), displaced blisters were detected (Figure 6). This suprabasal appearance suggests that epidermal migration had occurred beneath blisters, displacing them upwards. Remarkably, in both cases these displaced blisters were lined on the inside with LN-332 (Figure 6c and e) and surrounded by keratin 14-positive cells (Figure 6d and f), consistent with their derivation from blisters that had formed at the basal layer through BM rupture, and were then displaced during healing. Presumably, these displaced blisters are completely pushed out of the epidermis over time, explaining why they are not detected in adult mice. Interestingly, the timing for blister resolution differed between fibulin-2-null and α3-null mice. In contrast with P2 fibulin-2-null mice, P2 α3eKO mice showed only new blister formation, and new blister formation was not resolved in α3eKO mice until between P5 and P10 (data not shown).

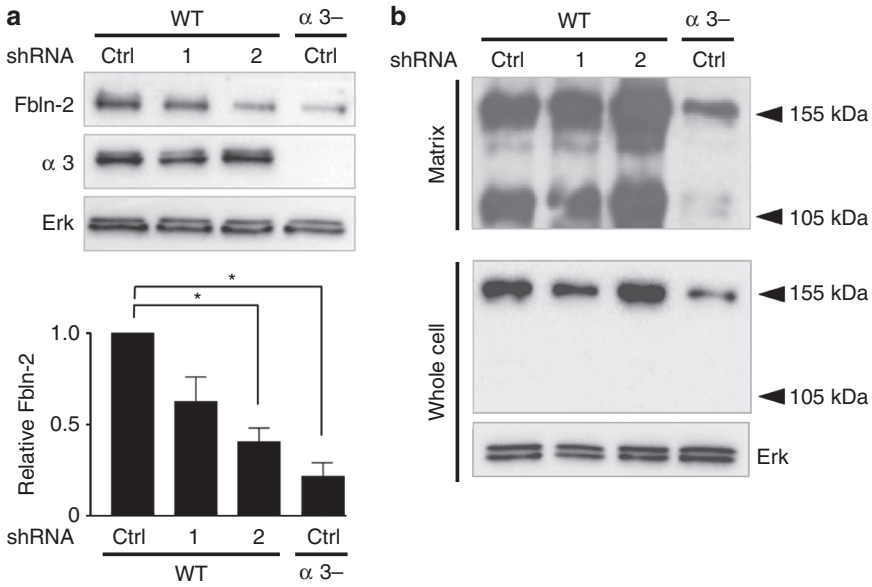


**Figure 5. α3β1-dependent modulation in laminin-γ2 processing is independent of fibulin-2 levels.** α3β1-expressing keratinocytes (wild type (WT)) were transduced with lentivirus expressing non-targeting short hairpin RNA (shRNA) (ctrl) or two distinct shRNAs that target fibulin-2 (Fbln-2; numbered 1, 2). As a control, α3β1-deficient keratinocytes (α3 − ) were tranduced with nontargeting shRNA (Ctrl). (**a**) Cell lysates were assayed by immunoblot to verify fibulin-2 knockdown. Anti-α3 confirmed the presence or absence of α3 integrin, and anti-Erk served as normalization control. Quantification of relative fibulin-2, normalized to Erk, as a proportion of levels in control shRNA-treated WT cells, is shown below. Data are mean ± SEM; $n = 3$; one-way analysis of variance, $P < 0.001$; Tukey's multiple comparison, $^*P < 0.05$. (**b**) Matrix preparations or whole-cell lysates were assayed by immunoblot with anti-laminin-γ2. As expected, both unprocessed (155 kDa) and processed (105 kDa) forms of laminin-γ2 were detected in matrix preparations, whereas only unprocessed laminin-γ2 was detected in whole-cell lysates. Erk served as a loading control for whole-cell lysates.

Together, these findings indicate that newborn fibulin-2-null mice and α3eKO mice each form blisters as a result of BM rupture. However, blisters appear to heal somewhat later in α3eKO mice, possibly due to loss of additional α3β1 functions that are important for epidermal adhesion.

## DISCUSSION

Integrin α3β1 is required for proper development of the cutaneous BM and maintenance of epidermal–dermal adhesion, as the absence of α3β1 in both mice and humans leads to the formation of skin blisters with BM splitting (DiPersio *et al.*, 1997; Has *et al.*, 2012). However, it has been unclear how the absence of α3β1 leads to a destabilized BM that eventually ruptures. Our current findings suggest that α3β1 promotes BM stability in part through induction of fibulin-2, and that this mechanism is important not only for epidermal adhesion during skin development but also to maintain adhesion of the neo-epidermis during adult wound healing. Given that fibulin-2 can bind laminins and other ECM proteins (Sasaki *et al.*, 1995; Utani *et al.*, 1997), there are several ways in which it may contribute to proper BM maturation. One possibility is that fibulin-2 is critical for initial assembly of BM through its interactions with other essential components. Indeed, fibulin-2 can bind entactin/nidogen, which facilitates the formation of ternary complexes with collagen IV, perlecan, and fibulin-1, which are important for BM assembly (Sasaki *et al.*, 1995). Another possibility is that fibulin-2 binding to the unprocessed γ2 chain of precursor LN-332 regulates its stable incorporation into newly forming BM, which may be important for BM maturation and strength (Utani *et al.*, 1997; Gagnoux-Palacios *et al.*, 2001; Sasaki *et al.*, 2001). The fact that LN-332 is the major adhesive ligand for α3β1 in the epidermis suggests the intriguing possibility of a feedback loop, wherein steps of BM assembly that require a fibulin-2/LN-332 complex might regulate α3β1 function during wound re-epithelialization. Interestingly, perinatal blisters seen in fibulin-2-null mice were typically smaller and showed signs of resolution sooner (i.e., by P2) than those that formed in α3eKO mice, indicating that α3β1 likely has other roles in BM organization in addition to modulating fibulin-2 expression.

It will be important in future studies to elucidate the mechanisms whereby α3β1 modulates fibulin-2 levels, as well as how fibulin-2 is eventually upregulated post blistering in the absence of α3β1. Interestingly, fibulin-2 is synthesized by both epidermal and mesenchymal cells in the skin/wound microenvironment (Pan *et al.*, 1993). Our concurrent microarray studies have identified fibulin-2 as an α3β1-dependent gene in MK cell lines (Missan D and DiPersio CM, in preparation), suggesting at least a partial contribution of fibulin-2 from the epidermal compartment in skin. However, immunostaining revealed fibulin-2 was also reduced deep in the dermis of α3eKO mice (Figure 4), where it co-localized with the fibroblast marker procollagen-1 (data not shown), consistent with published reports that dermal fibroblasts are a major contributor of fibulin-2 (Pan *et al.*, 1993). This staining pattern might suggest an additional mechanism of α3β1-dependent crosstalk from the epidermis to fibroblasts that modulates fibulin-2 production in the dermis. Such a mechanism would be consistent with our recent findings that α3β1 in keratinocytes promotes crosstalk

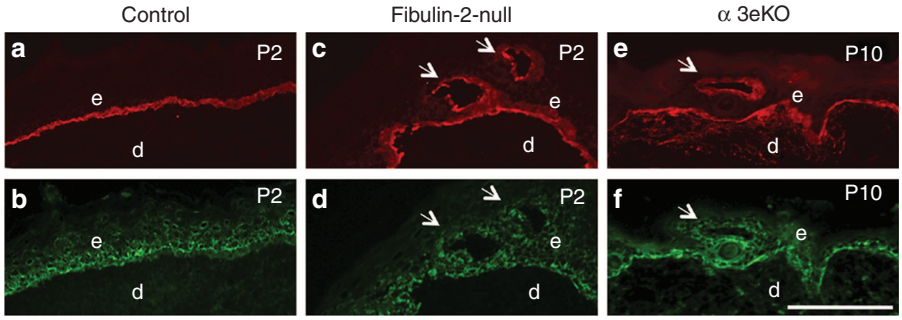


**Figure 6. Healing of blisters in fibulin-2-null mice or integrin α3 epidermal knockout (α3eKO) mice is evident from their displacement into the suprabasal layers of the epidermis.** Cryosections of paws from P2 control pups (**a**, **b**), P2 fibulin-2-null pups (**c**, **d**), or P10 α3eKO pups (**e**, **f**) were stained by double-label immunofluorescence with anti-laminin-332 (LN-332; red; **a**, **c**, **e**) and anti-keratin 14 to mark basal keratinocytes (green; **b**, **d**, **f**). Note that staining for LN-332 or keratin 14 is seen to line or surround, respectively, the displaced blisters, indicating that these blisters derived from the basal layer of the epidermis. Bar = 100 μm. Arrows, displaced blisters; d, dermis; e, epidermis.

to endothelial cells through induction of secreted pro-angiogenic factors (Mitchell *et al.*, 2009).

Interestingly, we observed that proteolytic processing of the laminin-γ2 chain is also impaired during wound healing in α3eKO mice and in α3-null keratinocytes, although this phenotype was independent of reduced fibulin-2. It seems likely that α3β1 regulates this processing through the modulation of extracellular proteases, such as BMP-1 or MT1-MMP, which have been shown to mediate laminin-γ2 chain cleavage (Amano *et al.*, 2000; Koshikawa *et al.*, 2004). Although *in vivo* roles of LN-332 processing are not yet fully understood, certain processing events are important for key linkages with other BM proteins (Aumailley *et al.*, 2003), and some can influence keratinocyte behavior. For example, differential processing of the laminin-α3 chain by tissue-type plasminogen activator or plasmin has been shown to alter epithelial cell motility (Goldfinger *et al.*, 1998, 1999). It will be interesting to determine whether laminin-α3 chain processing is similarly regulated by α3β1 during BM assembly. Indeed, both reduced levels of α3β1 and defective LN-332 processing have been reported in cylindroma skin tumors (Tunggal *et al.*, 2002), suggesting that integrin-dependent alterations in LN-332 processing should be further explored.

However, our current findings suggest that delayed γ2 processing in α3eKO mice is not causal to BM splitting, as the timing of blister formation during wound healing was not consistent with such a model, and we did not observe accumulation of unprocessed γ2 at sites of neonatal blistering. We speculate that delayed laminin-γ2 processing may actually be beneficial to blister healing in α3eKO mice, as the full-length γ2 short arm might allow for enhanced interaction with other ECM components, including residual fibulin-2. Indeed, we observed fibulin-2 to be eventually upregulated in α3eKO skin post blistering in both neonatal skin and adult wounds, which might have a stabilizing effect on the newly assembled BM that prevents subsequent blistering. Notably, the unprocessed γ2 chain within LN-332 may sustain cell adhesion rather than promote migration (Gagnoux-Palacios *et al.*, 2001). It is not currently known why the N terminus of laminin-γ2 is ultimately removed; however, retention of this region and the accompanying ECM interactions is not necessary to maintain a stable BM, as it is absent from fully processed LN-332 in mature BM of adult skin (Sasaki *et al.*, 2001; Aumailley *et al.*, 2003). Processed laminin-γ2 may poise the epidermis in a more ''migration-ready'' state for wound healing, consistent with evidence that unprocessed LN-332 supports adhesion (Gagnoux-Palacios *et al.*, 2001), whereas processed LN-332 may allow enhanced keratinocyte migration.

In summary, our study implicates fibulin-2 in α3β1-dependent assembly/integrity of the cutaneous BM and epidermal–dermal adhesion during skin development, and it extends this role for α3β1 to epidermal regeneration during adult wound healing. It seems likely that a role for fibulin-2, and possibly other fibulin family members, in promoting BM stability extends beyond the skin to other tissues. Indeed, fibulin-1-null mice die perinatally with severe defects in the BMs of many organs, including the kidney and lung (Kostka *et al.*, 2001). Interestingly, mice with global deletion of the integrin α3 subunit also display developmental defects in the kidney and lung, which include BM disorganization (Kreidberg *et al.*, 1996), and human patients with loss-of-function mutations in the *ITGA3* gene display kidney and lung defects in addition to skin blistering (Has *et al.*, 2012). Therefore, it is intriguing to speculate that a role for integrin α3β1 in regulating fibulins may be generally important for maintaining BM assembly and mechanical integrity of epithelial–stromal junctions.

## MATERIALS AND METHODS

### Mice

Epidermis-specific α3KO (α3eKO) mice are homozygous for a floxed α3 allele ($\alpha3^{flx/flx}$) and express a Cre recombinase transgene under control of the epidermis-specific keratin 14 promoter (K14-Cre), as previously described (Mitchell *et al.*, 2009). PCR genotyping of α3eKO mice (i.e., genotype K14-Cre:$\alpha3^{flx/flx}$) or control littermates that lack the K14-Cre transgene (i.e., genotype $\alpha3^{flx/flx}$) was performed as described (Mitchell *et al.*, 2009). The absence of α3β1 from the epidermis of α3eKO mice was routinely confirmed by immunostaining for the α3 subunit (Mitchell *et al.*, 2009). The generation and genotyping of fibulin-2-null mice has been described (Sicot *et al.*, 2008). Mouse studies were approved by the

Institutional Animal Care and Use Committee at Albany Medical College or Alfred I. duPont Hospital for Children.

### *In vivo* wounding and acquisition of neonatal tissue

Adult mice (6–10 weeks of age) were anesthetized and shaved, and four full-thickness wounds were made on the back using a sterile 4-mm biopsy punch, as described (Mitchell *et al.*, 2009). After 1, 5, 10, or 20 days of healing, mice were euthanized by $CO_2$ narcosis and wounds were surgically excised and bisected. Neonatal limbs were isolated from 0-, 2-, 5-, or 10-day-old pups following euthanasia. Two of four wounds and/or one forelimb and one hindlimb from each animal were frozen in optimal cutting temperature, while the remaining wounds and/or limbs were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (5 μM), and stained with hematoxylin and eosin.

### Immunofluorescence

Frozen sections (10 μM) were rehydrated in phosphate-buffered saline with 0.2% Tween-20 for 10 minutes, blocked in 10% heat-inactivated goat serum and 5% milk in phosphate-buffered saline for 1 hour, then stained with the following rabbit polyclonal antisera: anti-α3 integrin or corresponding pre-immune serum (1:100; DiPersio *et al.*, 1995); anti-LN-332 (1:200; Abcam, Cambridge, MA), anti-entactin/nidogen (1:1000; Abcam), anti-LNγ2L4m (1:1,000; Sasaki *et al.*, 2001), and anti-fibulin-2 (1:2,000; Pan *et al.*, 1993). Sections were co-immunostained in some cases with mouse monoclonal anti-cytokeratin 14 (1:500; Abcam). Secondary antibodies were fluorescein-conjugated goat anti-mouse IgG (1:250; Pierce, Rockford, IL) or Alexa Fluor 594 goat anti-rabbit IgG (1:250; Molecular Probes, Eugene, OR), as appropriate. Images were collected on a Nikon Eclipse 80i using a Spot camera (Diagnostic Instruments, Sterling Heights, MI).

### Cell culture and western blotting

MK cell lines that express or lack integrin α3β1 were derived previously (DiPersio *et al.*, 2000a; Iyer *et al.*, 2005). Total lysates were prepared in cell lysis buffer (Cell Signaling Technology, Beverly, MA) and protein concentrations determined using the BCA Protein Assay Kit (Pierce). Equal protein was resolved by nonreducing 7% SDS-PAGE and assayed by immunoblot using rabbit polyclonal antiserum against fibulin-2 (1:1,000; Pan *et al.*, 1993), α3 integrin (1:1,000; DiPersio *et al.*, 1995), or Erk-2 (1:1,000; Santa Cruz Biotechnology, Dallas, TX), followed by horseradish peroxidase–conjugated goat anti-rabbit IgG (1:1,000; Cell Signaling Technology). ECM fractions were prepared from near-confluent MK cultures grown for 3–4 days on noncoated dishes in keratinocyte growth medium (DiPersio *et al.*, 2000a) supplemented with 4 mM $CaCl_2$. Following removal of cells with 1 mM EDTA, matrix was scraped into DOC buffer (2% sodium deoxycholate, 20 mM Tris-Cl, pH 8.8, and 2 mM each of phenylmethylsulfonyl fluoride, EDTA, iodoacetic acid, and N-ethylmaleimide), as described (Wierzbicka-Patynowski *et al.*, 2004). The DOC-insoluble matrix fraction was solubilized in 4% SDS/reducing sample buffer, and equal volumes were assayed by immunoblot with anti-laminin-γ2 (1:200; Santa Cruz Biotechnology), followed by horseradish peroxidase–conjugated donkey anti-goat IgG (1:1,000; Santa Cruz Biotechnology). Chemiluminescence was performed using SuperSignal Kit (Pierce), then visualized using Bio-Rad ChemiDoc MP imaging system with Image Lab software (Bio-Rad, Hercules, CA).

### shRNA-mediated suppression of fibulin-2

Lentiviral vectors (pGIPZ) containing a non-targeting shRNA or fibulin-2-targeting shRNA (shRNA 1, V3LMM 515480; shRNA 2, V3LMM 26531; Thermo Scientific Open Biosystems, Lafayette, CO) were cotransfected into 293FT cells with packaging plasmids pCMV-dR8.2 and pCMV-VSV-G. MK cells were infected with viral particles along with antennapedia peptide (Anaspec, Fremont, CA), then selected in 10 $\mu g\,ml^{-1}$ puromycin to generate stably transduced populations.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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